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13. SUPPLEMENTARY NOTES

14. ABSTRACT The purpose of the award is to compare mammary glands from wildtype and IL-10 knockout mice at critical stages of development. IL10-knockout mice were imported and maintained in University at Buffalo lab animal specific pathogen-free facility. During the first year of the funding (July 06-July 07), we established protocols in our lab for genotyping, mammary gland whole mount preparation and the preparation of paraffin sections of mammary glands. We then mated IL10+/- male with IL10+/- females to generate 144 experimental female mice with +/+ or -/- genotype (72 each) from 80 litters. After excising and processing the glands at 5 critical stages of mammary gland development as described in the grant, we developed data analysis methods to perform the planned comparison. There were no differences between the wildtype (IL10+/+) and IL10-/- mice at up to age day 55. At age day 80 and 150, IL10-/- mice had less terminal end buds suggesting an alteration in mammary gland development. Part of the results are presented in the 2008 Era of Hope meeting (meeting abstract attached).

15. SUBJECT TERMS
IL-10, mammary gland

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Introduction

The award aims to determine the relationship between anti-inflammatory IL-10 and mammary gland development. To achieve the goal, we proposed to compare mammary glands from wildtype and IL-10 knockout female mice at different stages of development: d 21, 55, 80 and 150 of age and 2 days after giving birth.

Body

There are two major tasks: first, to breed and raise wildtype and IL-10 knockout female mice by mating IL-10 heterozygous female with IL-10 heterozygous male; second, to develop essential techniques for mammary gland analysis so that we can compare mammary glands at the age of interest.

Task 1: animal breeding. University at Buffalo lab animal specific pathogen-free facility (where the experimental mice are housed) was going through major renovation in the summer/fall of 2006, and then major reorganization of animal care in Feb-May of 2007. Since then, we have mated IL10+/- male with IL10+/- females to generate 144 experimental female mice with +/+ or -/- genotype (72 each) from 80 litters. In order to ensure that the comparisons were made between mice at the same stage in the estrus cycle, postmortem vagina smear was performed on day 55, 80 and 150 mice.

Task 2: mammary gland comparison. We first established the techniques that are needed for mammary gland harvesting and processing in our lab including whole mount preparation and the preparation of paraffin sections of mammary glands. We then developed computer software (ImageJ)-based methods to objectively performing quantitative characterization of the whole mount samples and paraffin sections (see below for details).

After excising and processing the 4th pair of mammary glands at 5 critical stages of mammary gland development, day 21, 55, 80 and 150 and 2 days after the delivery, we converted the images to digital files by using a high-resolution scanner (for whole mount slide) and a high-resolution digital camera attached to a microscope (for paraffin sections). Representative images of whole mount slides and hematoxylin/eosin-stained paraffin sections of each stage are included in the appendices (pp. 3-6). For the analysis of the mammary gland whole mount, we focused on the area from lymph node to the outer edge of the gland counting the number of peripheral terminal end buds and the number of peripheral side branches. The distance between nipple and the lymph node, the size of lymph node and the total ductal area are also measured using ImageJ. For analyzing the hematoxylin/eosin-stained paraffin sections, we used the 6th longitudinal section of each gland (4 μm per section from the top of the gland). Eight evenly spaced images surrounding the lymph node were captured from each slide. The extent of leukocyte infiltration was determined by the mean total nuclei of 8 areas. The size of white adipocytes was determined by measuring the adipocyte size of a five-cell cluster in the middle of each of the 8 areas and then obtain a mean of the 40 measurements (5x8).

Our initial analysis focused on the mice in estrus and the results are shown the appendices (pp. 3-6). The data were presented in the Era of Hope 2008 meeting (see appendices pp. 7-8). Although

the grant has ended, one graduate student is still performing analysis of the whole mount and paraffin section slides. Based on the analyses that have been completed, there were no differences between the wildtype (IL10+/+) and IL10-/- mice at up to age day 55. At day 80 and 150, IL10-/- mice had less terminal end buds suggesting an alteration in mammary gland development. In the literature, decreasing IL-10 was linked to breast cancer risk indirectly. Our data represent the first direct examination of the relationship between IL-10 and mammary gland development.

Key Research Accomplishments

Using IL10-knockout mice as the model, in our first set of data analysis, we found a link between IL-10 and mammary gland development. This was never reported before. Complete data analysis will help to determine the extent of the effect and the potential implication in breast cancer risk.

Reportable Outcomes

We presented in the Era of Hope 2008 meeting and plan to submit a manuscript at the completion of the second phase of data analysis.
An M.S. graduate student with the intended major of Biotechnology, Omkara Lakshmi M Veeranki, is currently continuing the data analysis. Although she is not supported by funding for the project, part of the project will be her M.S. thesis and she will be well trained for mammary gland analysis.

Conclusion

We have preliminarily identified a role of IL-10 in the mammary gland development using the IL10-knockout mouse model. Upon the completion of the data analysis, we intend to submit a manuscript and seek more grant support to continue the investigation of the link between IL-10 and mammanry gland development/mammary cancer risk.

References

None

Appendices

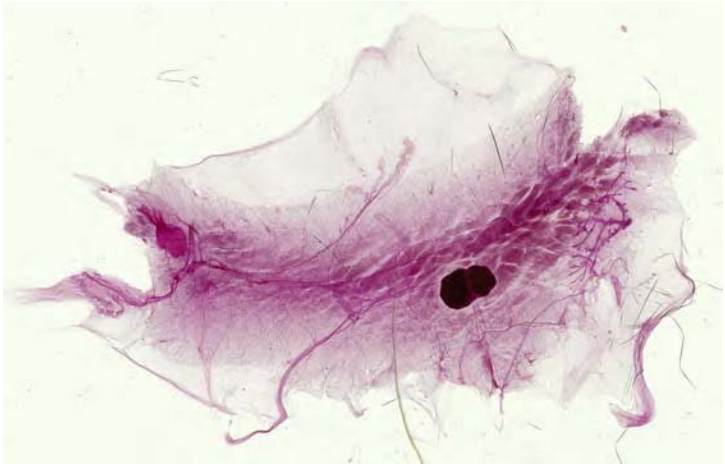
Summarized data presented in the Era of Hope 2008 meeting (pp. 3-6)
Era of Hope 2008 meeting abstract as appeared in the meeting proceeding (pp. 7-8)

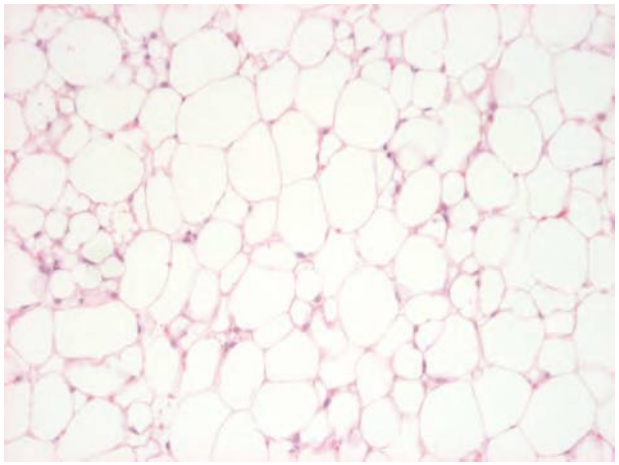
Day 21

Whole mount

paraffin sections

+/+





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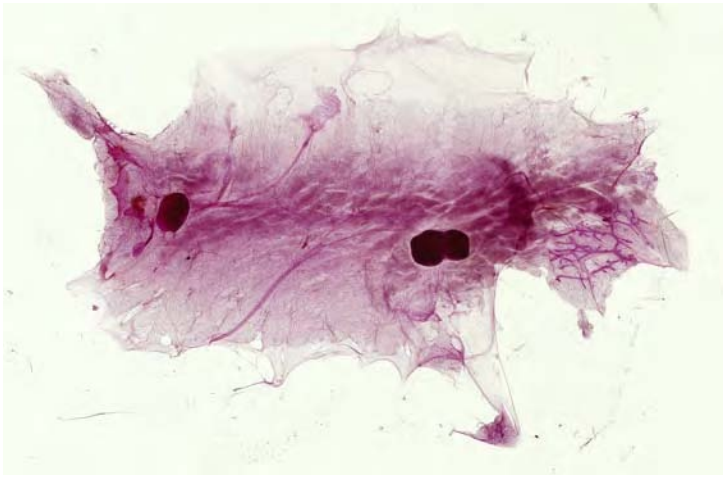

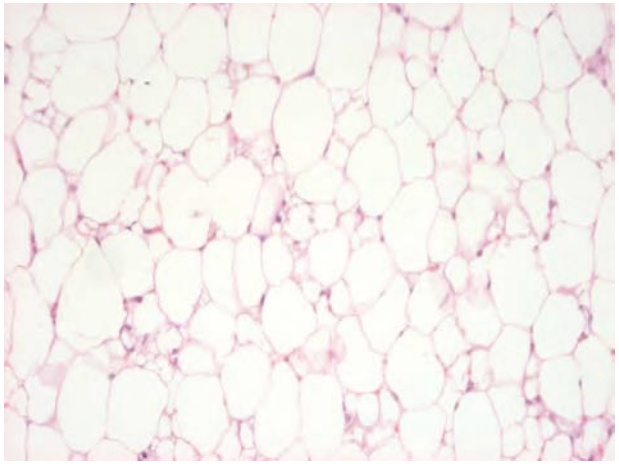

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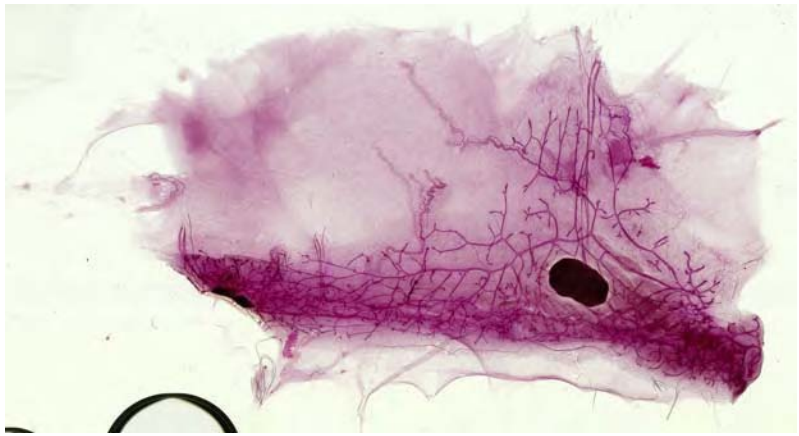
			Whole mount analysis				Paraffin section analysis	
D21	N	body weight (g)	total TEB	total ductal side branch	lymph node area (mm2)	BAT area (mm2)	WAT size (μm2)	total nuclei (per image)
+/+	8	8.5±2.2	27±13	5.6±3.5	1.83±0.29	37±11	1418±273	182±34
-/-	7	10.2±1.1	25±9	4.1±1.9	1.95±0.45	44±9	1389±291	183±32

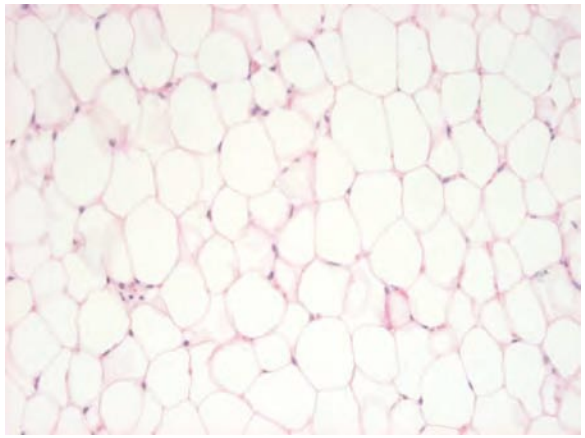
Day 55

Whole mount

paraffin sections

+/+





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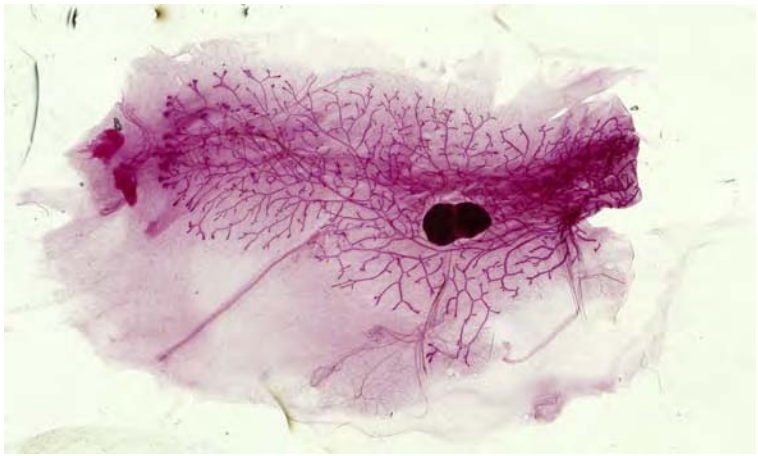
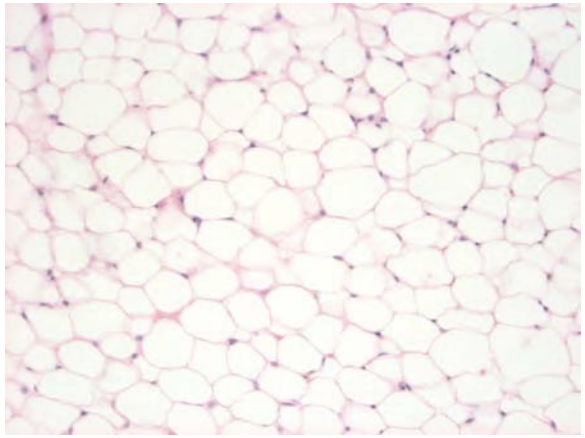




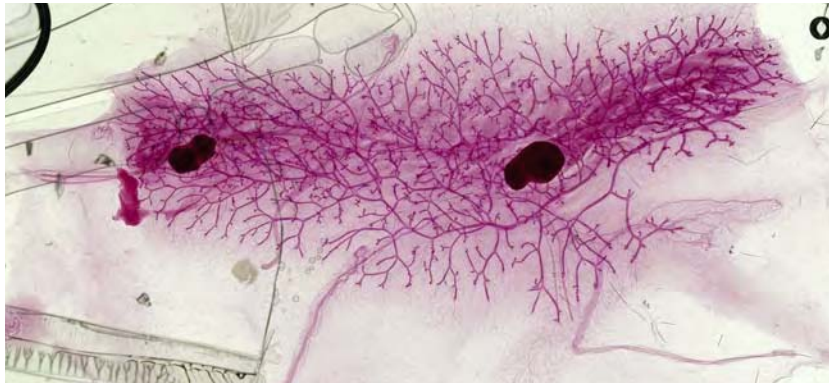
			Whole mount analysis						Paraffin section analysis			
D55	N	body wt (g)	peripheral branch	peripheral TEB	TEB/branch (%)	total ductal area (mm2)	TEB/ductal area	lymph node area (mm2)	nipple to node distance (mm)	WAT size (µm2)	total nuclei (per image)
+/+	7	20.5±1.5	148±76	118±80	76±12	112±18	1.06±0.74	3.27±0.62	6.89±2.33	1662±541	164±67
-/-	4	20.8±1.5	152±34	124±30	82±7	84±21	1.53±0.41	2.49±0.46	6.05±0.87	1347±272	152±23

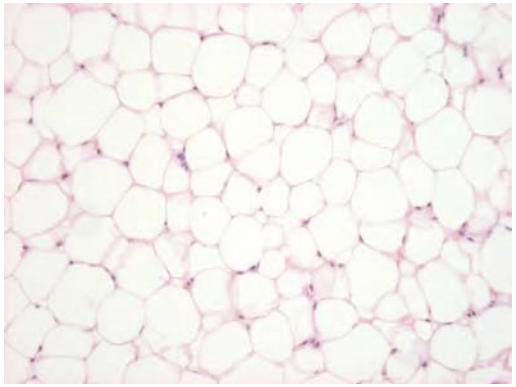
Day 80

Whole mount

paraffin sections

+/+





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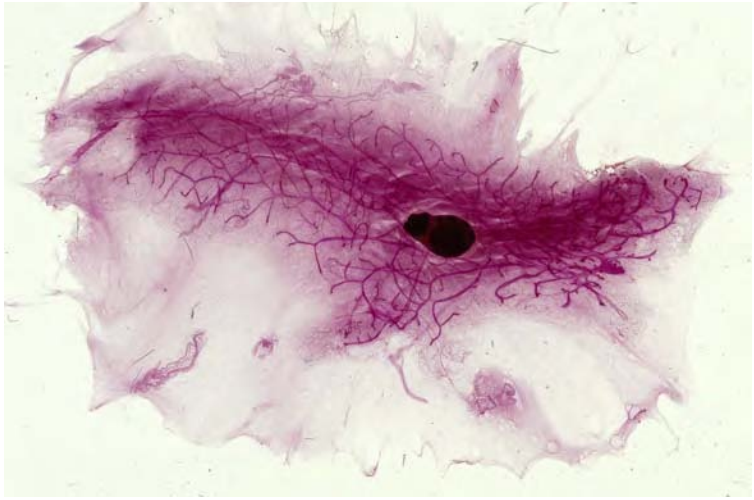
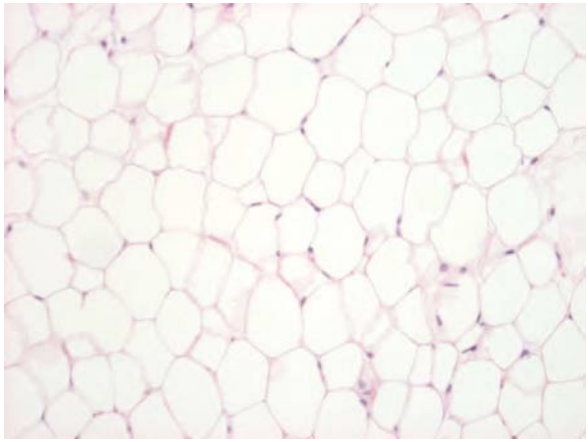




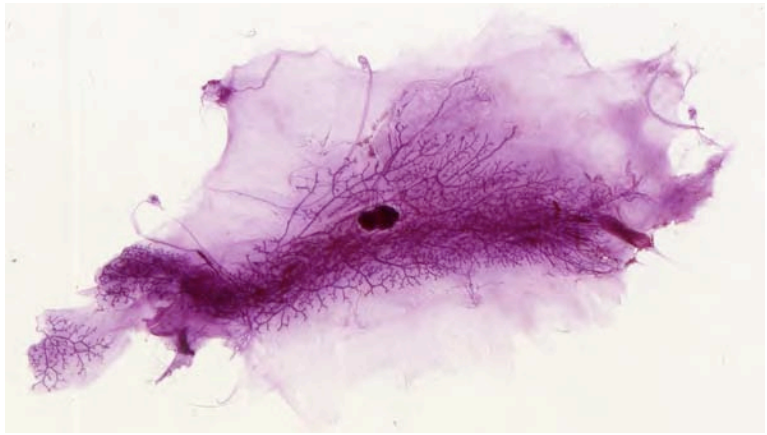
		Whole mount analysis								Paraffin section analysis	
D80	N	body wt (g)	peripheral branch	peripheral TEB	TEB/branch (%)	total ductal area (mm2)	TEB/ductal area	lymph node area (mm2)	nipple to node distance (mm)	WAT size (µm2)	total nuclei (per image)
+/+	4	21.4±1.6	159±76	123±82	73±17	160±76	1.40±0.84	2.43±0.60	9.37±4.68	1562±368	119±29
-/-	4	21.0±2.4	113±39	70±47	58±18	113±39	0.64±0.27	3.44±0.41	4.59±0.54	1690±398	133±39

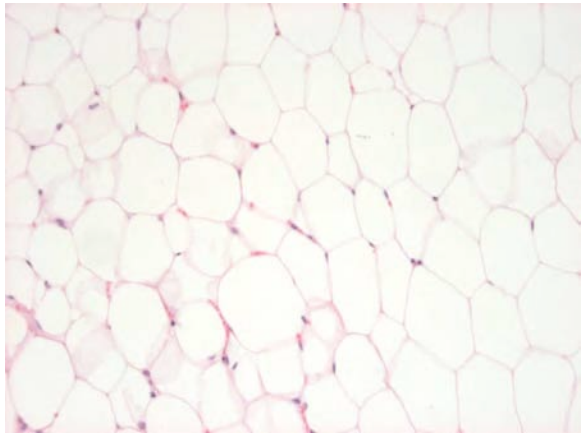
Day 150

Whole mount

paraffin sections

+/+





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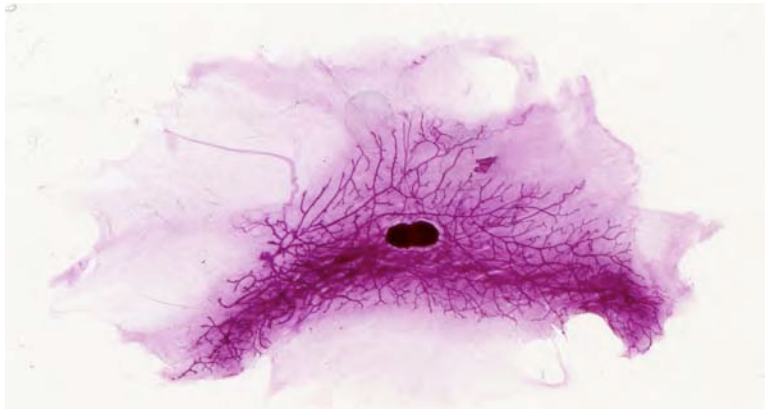

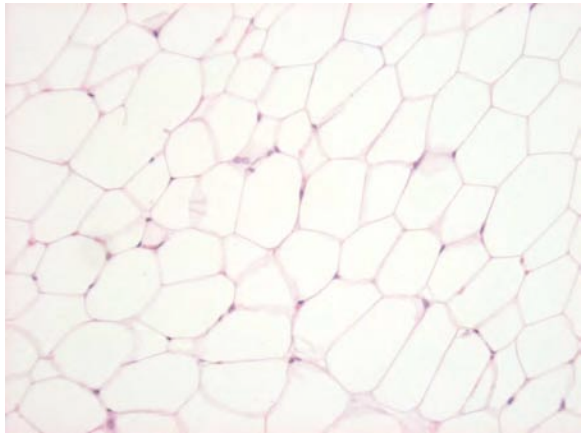

		Whole mount analysis						Paraffin section analysis		
D150	N	body wt (g)	peripheral branch	peripheral TEB	TEB/branch (%)	total ductal area (mm2)	TEB/ductal area	lymph node area (mm2)	WAT size (µm2)	total nuclei (per image)
+/+	4	25.5±4.7	104±32	55±21	52±6	120±54	0.57±0.31	2.38±0.36	1809±989	152±54
-/-	4	26.6±3.6	72±9	39±6	54±3	139±33	0.30±0.10	2.84±0.41	2779±1122	90±51

MAMMARY GLAND DEVELOPMENT

Poster Session P70

P70-1: CREATING A BIOREPOSITORY OF "NORMAL" TISSUE AND BIOMOLECULES FOR BREAST CANCER RESEARCH

Connie Rufenbarger
Catherine Peachey Fund

Introduction/Objective: In 1998 the National Cancer Institute published the report of the Breast Cancer Progress Review Group (PRG). The Executive Summary of PRG states: "…it is now clear that a more complete understanding of the normal mammary gland at each stage of development—from infancy through adulthood—will be a critical underpinning of continued advances in detecting, preventing, and treating breast cancer." There are currently few annotated collections of "normal" breast tissue. Research using normal tissue is essential to understanding the causes of cancer, discovering biomarkers, and identifying targets for prevention/treatment. The goal of this project, which was led, designed, implemented, and overseen by the Catherine Peachey Fund, in collaboration with the IU Simon Cancer Center; is to create a large repository of samples of blood, serum, plasma, saliva, and breast tissue from women with no clinical evidence of breast cancer which is collected and stored following the *NCI Best Practices for Biospecimen Resources.*

Description: The Catherine Peachey Fund, Inc., is an Indiana-based, nonprofit, consumer organization that supports breast cancer research. The Amelia Project is the Peachey Fund's annual scientific meeting, which partners laboratory researchers and clinicians with consumers. The normal tissue bank originated at the Amelia Project. Researchers attending the meeting identified the lack of access to "normal" breast tissue samples as one of the most significant roadblocks to progress in breast cancer research. The shared experience of the group was that "normal" samples were not available in sufficient numbers and that what was available did not meet the required quality standards for reliability. Consumer representatives present realized that this was a need that they could address, and they spearheaded and funded an unprecedented collaboration among consumers, clinicians, basic scientists, volunteers, and donors who took up this challenge. To the best of our knowledge, this is the first biospecimen bank in the world, the purpose of which is to collect specimens from individuals without disease.

It took 3 years to address ethical and IRB issues as well as to develop the necessary infrastructure. Donors fill out a four-page questionnaire of comprehensive medical information. The annotation is linked to the tissue sample through a barcode system. Data is stored in an Oracle database that is HIPAA compliant. The database is available via the World Wide Web to researchers across the world who can query it to determine if there are specimens available that have the potential to facilitate their research.

The Bank is governed by a steering committee made up of a consumer representative, a medical oncologist, and a surgeon. Consumer representatives serve on all standing committees of the Bank

Summary of Results: The Bank at present contains almost 2,500 specimens including breast core biopsies from 178 women. Outreach to minority populations has been a priority of the Bank, 14% of specimens are from African-American women recruited at Indianapolis Black Expo. Research utilizing the samples is robust and has resulted in poster presentations at the San Antonio Breast Cancer Symposium, publications, and multiple collaborations across the country. The Bank has been recognized by Susan G. Komen for the Cure who has provided a 1-year, one million dollar grant to support the Bank.

This work was supported by The Catherine Peachey Fund, Inc.; Indiana University School of Medicine; and The General Clinical Research Center.

P70-2: MAMMARY GLAND DEVELOPMENT IN IL-10 KNOCKOUT MICE

Shiu-Ming Kuo and Patricia A. Masso-Welch
State University of New York, Buffalo

Several previous studies suggest that IL-10 may influence mammary carcinogenesis. Mammary epithelial cells and breast cancer cells can both respond to IL-10. IL-10 has been shown to reduce growth and metastases of malignant mammary tumor cells in immunocompetent and immunocompromised mouse hosts. In another mouse model, pathogen exposure increased mammary gland tumor development and an adaptive transfer of IL-10-competent TR cell reduced cancer risk. In the human, an IL-10 gene promoter polymorphism that increases IL-10 production has been associated with decreased breast cancer risk. This polymorphism also increased breast cancer survival after autologous bone marrow transplantation. To test the hypothesis that IL-10 loss will alter the mammary gland in ways that increase breast cancer susceptibility, we are examining the effect of IL-10 gene knockout on mammary gland development and differentiation. Mammary gland comparisons will be made between IL-10 knockout virgin female mice and their wild-type littermates at critical points of mammary gland development, including age d21, d55, d80 and d150 of age as well as 2 days after delivery. We have now compared 5 pairs of mice at d21 (weaning). At this stage, IL-10 knockout did not significantly affect mammary gland structure. In mammary gland wholemount analysis, there were no differences in the number of terminal end buds, the number of ductal side branching, nor the size of lymph node. In the analysis of hematoxylin and eosin-stained paraffin thin sections, there was no difference in leukocyte abundance in the stroma. Initial gross and histologic analysis of the adipose fat pad did not reveal any significant difference in structure. D21 IL-10 knockout mice and control mice had similar average size of white adipocytes (determined in paraffin sections) and similar area of brown adipose tissue (determined in mammary gland wholemount). To summarize, at day 21 of development, there was no significant difference in gland development between IL-10 knockout and wild-type mice. Ongoing studies are addressing the effects of IL-10 knockout on the development and differentiation of the mammary gland during and post-puberty, time points when leukocytes have been shown to play a role in mammary gland branching morphogenesis.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0645.

P70-3: GLOBAL CHANGES IN PATTERNING OF HISTONE MODIFICATIONS MARK MAMMARY GLAND MATURATION AND DIFFERENTIATION

Monique Rijnkels, Violeta Chen, David Edwards, and Daniel Medina
Baylor College of Medicine

An early full-term pregnancy has been shown to have a protective effect with regard to the risk of developing breast cancer. It has been suggested that parity-related protection occurs through a change in cell fate, affecting a specific population of mammary epithelial cells. This change in cell fate most likely occurs through an epigenetic mechanism, thus pointing to a direct role for chromatin remodeling in this process. Histone-tail modifications are marks for different chromatin structural states that correlate with development. We hypothesized that there are changes in histone modifications correlating with the protective effect of full-term pregnancy and marking cells with a different fate. To test this hypothesis in an animal model, we (1) determined that unique changes in global histone modification staining patterns and levels in mammary epithelial cells of the mouse mammary gland correlated to full-term pregnancy and (2) tested the significance of global histone modification patterns in samples from hormone pretreated mice and age-matched virgins (AMV) (6 months) that have been treated with/without carcinogen (DMBA). Staining protocols were established for virgin, pregnant, lactating, and involuted mammary gland tissue using antibodies against specific histone modifications. We detected developmental stage-specific nuclear staining patterns for the investigated histone modifications. It showed that the functional differentiation of mammary epithelial cells occurs in concert with a profound reorganization of the chromatin. We did not detect differences in staining patterns or levels between mammary glands after full-term gestation and lactation and age-matched virgins nor did we detect changes correlating with estrogen and progesterone treatment in a tumor prevention model. These findings suggest that the gross changes in histone modification patterning and levels observed during functional differentiation of the mammary gland from virgin to lactation do not persist after lactation has ceased. This illustrates the dynamic nature of the observed changes in chromatin and histone modification patterning and the remarkable plasticity of the mammary epithelial cells. It does not rule out that local chromatin changes, which are not detected by the global analysis employed here, are occurring and important in pregnancy-related protection. Insight in the changes in the compaction of the DNA in breast cells after a full-term gestation can help in understanding the changes that contribute to parity-related protection from cancer. This can lead to new approaches in diagnosis and treatment of this disease that affect millions of women in this country.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-05-1-0456.

P70-4: MAMMARY INTRADUCTAL FOAM CELLS ARE BONE MARROW DERIVED AND ARE RECRUITED IN RESPONSE TO BOTH PHYSIOLOGICAL AS WELL AS NEOPLASTIC STIMULI

Sanford H. Barsky, Yi Xiao, Yin Ye, and Kurtis Yearsley
Ohio State University

Intraductal "foam cells" are the most commonly encountered cells in spontaneous nipple discharge, nipple aspirate fluid, and ductal lavage yet their origin and significance remain a mystery. These cells increase in pregnancy and other conditions of ductal ectasia and obstruction. They frequently surround DCIS and other intraductal proliferations. Our previous immunocytochemical studies with macrophage (CD68, lysozyme), epithelial (cytokeratin, estrogen receptor) and myoepithelial (smooth muscle actin, CALLA, maspin) markers indicated that foam cells are of macrophage lineage and terminally differentiated (negative Ki-67 and PCNA). These foam cells often ingest both endogenous as well as exogenous substances. Because these macrophages are observed only intraductally and because their appearance resembles lactating and vacuolated epithelial cells, their origin had been presumed to be of ductal lining epithelium. However, our previous studies utilizing bone marrow transplantation of donor marrow from female GFP-transgenic C57 black mice into sublethally irradiated female C57 black mice recipients rendered pseudopregnant revealed that the mammary foam cells were of donor bone marrow origin. Mice exhibiting successful bone marrow engraftment of at least 50% donor marrow were identified and made pseudopregnant with a combination of estradiol, progesterone, and estriol (2.5 mg) 21-day release pellets. After this time period and following euthanasia, their mammary fat pads were excised and examined. The presence of GFP-containing intraductal foam cells was found.